GENOTYPING PROTOCOL

MUTANT MOUSE RESOURCE & RESEARCH CENTER: UC DAVIS

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Protocol Name: **CR10673 Tbc1d5 Exon14+1(G>C) HDR** Stock #: 66781

Reagent/Constituent	Volume (µL)
QuantiTect Multiplex PCR Master Mix **Cat No./ID 204541**	5.0
Water	3.4
Target Probe mix	0.3
-21 µM Mutant Forward Primer	
-21 µM Mutant Reverse Primer	
-7 µM Mutant probe	
TCRD (endogenous control) mix	0.3
-21 µM TCRD Forward primer	
-21 µM TCRD Reverse Primer	
-7 µM TCRD probe	
Sample	1.0
TOTAL VOLUME OF REACTION:	**10.00 µL**

Comments on protocol:

Protocol may work with other DNA extraction methods. Reference: ABI User Bulletin #2 and #5 (updated 10/2001) for multiplex in same tube and validation of each assay to match relative efficiencies of reference and target primer/probe combinations. Also reference: Rapid and accurate determination of zygosity in transgenic animals by real-time quantitative PCR. TransRes (2002).

Strategy:

Steps	Temp (ºC)	Time (m:ss)	# of Cycles
1. Initiation/Melting	95	15:00	**1**
2. Denaturation	95	0:30	**40x**
3. Annealing/Elongation	60	1:00	**40x**
4. To step 2 for 40 cycles			

Primers:

	Name	Nucleotide Sequence (5' - 3')
1.	TCRD Forward Primer	CAGACTGGTTATCTGCAAAGCAA
2.	TCRD Reverse Primer	TCTATGCCAGTTCCAAAAAACATC
3.	TCRD MGB VIC Probe	VIC-ATTATAACGTGCTCCTGG-MGB
4.	TM_Tbc1d5-F	GCCTCAACCTGAGCTTAGTCG
5.	TM_Tbc1d5-R	TGGACTATGGTAAAGTGAGACATACTTAG
6.	Tbc1d5 BHQ-1 FAM Probe	Fam-pdU-A-pdU-A-pdU-pdC-pdC-G-pdC-G-pdC-pdU-G-pdC-pdU-pdU-pdC-pdU-A-BHQ-1

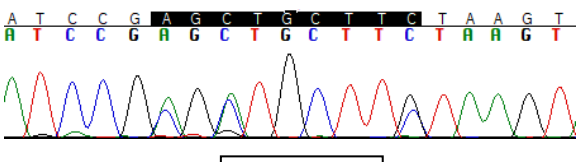
Allele Description: The mouse Exon14+1(G>C) model was created using optimized CRISPR Cas9 KI technology utilizing Ribonucleoprotein (RNP) in the presence of a synthetic single strand DNA repair template harboring the desired KI. Zygotes were electroporated and subsequent progeny were screened for the presence of the correctly targeted allele via homology directed repair (HDR). The KI nucleotide is 5 bp from cleavage site and two silent protospacer mutations are engineered into ssODN to protect the HDR derived locus from auto-cutting by Cas9. Key progeny were sequence confirmed.

gcctcaacctgagcttagtcgactatgtcttcacagccatgctactctatatccg**C**g**C**tgctt**C**taagtatgtctcactttaccatagtccactaggtacccat

WT G>C KI

Sample	ΔCt	Genotype
Tbc1d5-ntc		No Rxn
Tbc1d5-wt	2.43	WT
CR10673-40	-0.19	Het
CR10673-42	-0.12	Het

Note: Homozygous animals will have a deltaCt around -1.3 (1 Ct less than heterozygous animals).



Het

AgAtgcttG
WT

CgCtgcttC
KI

PCR protocol developed by MMRRC at University of California, Davis

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Alternative Genotyping Protocol
Standard PCR and Sequencing

Protocol: ***GoTaq® G2 Colorless Master Mix(Promega)***

Reagent/Constituent	Volume (µL)
Water	5.0
GoTaq® G2 Colorless Master Mix,2X	7.5
Primer 1. (stock concentration is 20µM) IVF	0.5
Primer 2. (stock concentration is 20µM) IVR	0.5
DNA (example) extracted w/ "Qiagen DNeasy columns or other similar silica based kits"	1.5
TOTAL VOLUME OF REACTION:	**15.0 µL**

Comments on protocol:

- Protocol may work with other DNA extraction methods.
- When crossing Alg13 and Glt28d2 the Taqman picks up the "other" mutation. Sequencing will be needed if mixed.

Strategy:

Steps		Temp (ºC)	Time (m:ss)	# of Cycles
1. Initiation/Melting	HOT START? ☐	94	2:00	**1x**
2. Denaturation		94	0:10	
3. Annealing	steps 2-3-4 cycle in sequence	65 (↓1ºC/cycle)	0:30	**10x**
4. Elongation		68	2:00	
5. Denaturation		94	0:15	
6. Annealing	steps 5-6-7 cycle in sequence	55	0:30	**25x**
7. Elongation		68	2:00 (↑20sec/cycle)	
8. Finish		4	∞	n/a

Primers:

Name	Nucleotide Sequence (5' - 3')
1. CR_Tbc1d5_ex14-IVF	ATTGTCTTGCTTTGACACATGATGC
2. CR_Tbc1d5_ex14-IVR	AGCACCCCAAGGTAGTTCTGAGGA
Tbc1d5_ex14-seqF	GGTGCGGCTGCTGTTTGG

Electrophoresis Protocol:

Agarose: **1.5%** : **90**

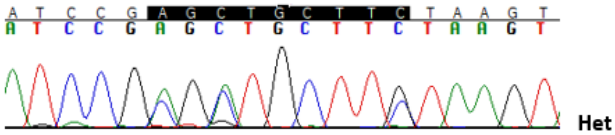
Estimated Running **90** min.

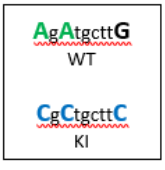
Primer Combination	Band (bp)	Seq Primer
1 & 2	819	Tbc1d5_ex14-seqF

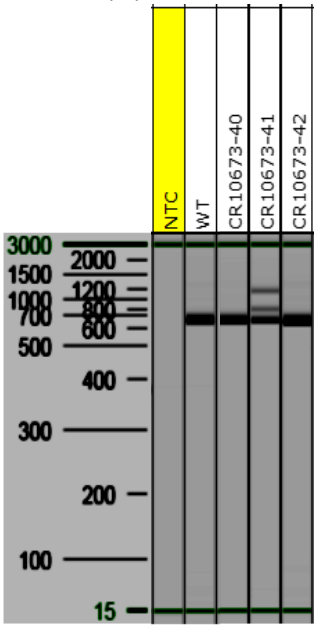
Sequencing across Exon 14 to verify the HDR using standard PCR and PCR purification is used to determine the presence of HDR.

Note: Sequence the WT-sized band. Pup #41 was not sequenced as previous TM data indicated that this pup did not have HDR. It is necessary to sequence all WT-sized bands to verify HDR when using only standard PCR.

Sample	Genotype
CR10673-40	Het
CR10673-42	Het







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